Design of Primers for Polymerase Chain Reaction Amplification of Mitochondrial DNA of Coregonine Fishes

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DESIGN OF PRIMERS FOR POLYMERASE CHAIN REACTION AMPLIFICATION OF MITOCHONDRIAL DNA OF COREGONINE FISHES

by

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ABSTRACT

Hisanaga, T.L., R. Banjo, T.J. Carmichael, and J.D. Reist. 2003. Design of primers for polymerase chain reaction amplification of mithochondrial DNA of Coregonine fishes. Can. Tech. Rep. Fish. Aquat. Sci. 2487: iv + 22 p.

Primers for the polymerase chain reaction were designed to amplify the entire mitochondrial genome of various Coregonines. The mitochondrial genome of *Coregonus lavaretus* (Genbank accession no. NC_002646) was used as the template for primer design. The mitochondrial genomes for a variety of fish determined to be representative of the Coregonines (Appendix 1) were successfully amplified. This basic step provides the basis for sequencing the complete mitochondrial genome of Coregonine fishes, thus allowing for taxonomic and phylogenetic studies.
~~Keywords: PCR product, mitochondrial molecule, coding and noncoding regions, sequence alignment, taxonomic identification~~.

Key words: PCR, direct sequencing, taxonomic identification, phylogenetic relationships, Coregonus, Prosopium, Salvelinus.

RÉSUMÉ

Hisanaga, T.L., R. Banjo, T.J. Carmichael, and J.D. Reist. 2003. Design of primers for polymerase chain reaction amplification of mithochondrial DNA of Coregonine fishes. Can. Tech. Rep. Fish. Aquat. Sci. 2487: iv + 22 p.

On a conçu des amorces de la réaction en chaîne de la polymérase en vue d'amplifier le génome mitochondrial intégral de divers Corégonidés en se servant du génome mitochondial de *Coregonus lavaretus* (numéro d'accession de GenBank NC002646) comme matrice. On a ainsi réussi à amplifier les génomes mitochondriaux de divers poissons identifiés comme étant des Corégonidés (annexe 1). Cette étape fondamentale servira d'assise au séquençage du génome mitochondrial intégral de poissons de cette famille, ce qui permettra de mener des études taxinomiques et phylogénétiques.

Mots clés : PCR, séquençage direct, identification taxinomique, relations phylogénétiques, Coregonus, Prosopium, Salvelinus.

INTRODUCTION

Mitochondrial DNA (mtDNA) is maternally inherited DNA that replicates independent of nuclear DNA, and is not subject to recombination. The rate of mutation of mtDNA is constant and 5-10 times greater than most regions of nuclear DNA (Moritz *et al.*, 1987), rendering it extremely useful as a molecular chronometer in phylogenetic studies. Furthermore, consistent differences in mtDNA sequences across taxa, if they occur, may be useful for identifying closely related taxa. Single base changes can be detected among taxa using such techniques as restriction fragment analysis and direct sequencing. As well, comparisons can be made if additions and deletions of bases of significant size exist. These can be detected by simply determining the length of a polymerase chain reaction product. The utility of these techniques for elucidating differences in mtDNA among species, and for using such information to determine taxonomic uniqueness and relationships is well established (Bernatchez *et* al., 1991, Brzuzan, 2000, Fournier-Lockwood *et al.*, 1993, Reist *et al.*, 1998).

The polymerase chain reaction (PCR) results in rapid, exponential amplification of minute amounts of DNA, allowing for sufficient quantities of DNA to be analyzed using the sequencing and related techniques (Baumforth *et al.*, 1998). The PCR process requires short oligonucleotide primers to both start and direct the amplification of a region of interest. In order to achieve high fidelity amplification, the primers must: 1) bind to the target DNA sequence with high specificity, 2) bind at strictly one site along the target (i.e., be unique over the entire mitochondrial genome), and 3) anneal to the target DNA at a fairly high temperature (Baumforth *et al.*, 1998, Beckenbach, 1991). This will prevent the amplification of artifacts while ensuring an abundance of the desired product. It is also desirable to design primers that bind to areas which are highly conserved areas, making them useful for PCR amplification in a wide variety of species. Primers are designed for both the plus and minus strands of the mitochondrial DNA molecule. This brackets the desired region, and ensures the PCR product is uniform in size so that when the PCR product is later used as a template for DNA sequencing, the integrity of the sequence achieved is greater, and a greater number of nucleotides can be accurately determined.

The mitochondrial DNA of the Salmonidae, of which the Coregonines form a distinct subfamily, is circular and approximately 17 000 nucleotide pairs in length, although considerable variation in length exists among and sometimes within species (Brzuzan, 2000). Mitochondrial DNA codes for ribosomal RNAs and transfer RNAs used in the mitochondrion, and contains only 13 recognizable genes that code for polypeptides (Beckenbach, 1991). Each of the genes code for a different polypeptide that makes up part of the electron transport chain in the inner mitochondrial membrane: NADH dehydrogenase subunits 1-6 and 4L, cytochrome oxidase subunits 1-3, cytochrome b, and ATPases 6 and 8. There also exists a region of higher variability within the mitochondrial genome, the control region, which is also known as D-loop (Lee *et al.*, 1995). Since this region contains the origin of replication, it is shown to be more liable to contain tandemly repeated sequences (Brzuzan, 2000), and a greater number of substitutions as well as insertions and deletions compared to the remaining regions of mtDNA, such as the gene-coding regions (Lee *et al.*, 1995).

Requirements envisioned as part of the ~~impending~~ species-at-risk legislation for confirming distinctness and identification of fish taxa, particularly Coregonines that exhibit some levels of risk (Sue Cosens, Freshwater Institute, 501 University Crescent, Winnipeg, MB, Canada, R3T 2N6, personal communication), suggested the need for applying genetic techniques such as direct sequencing that have recently become available. The framework for sequencing studies is outlined in Figure 1 with the portion customized in this report indicated. Previous work regarding direct sequencing of mtDNA conducted within the Freshwater Institute Science Laboratory fish genetics laboratory focussed primarily upon a small portion of the entire mitochondrial genome, approximately 400 bases of the D-loop (Brown-Gladden *et al.*, 1995, Reist *et al.*, 1998). This work demonstrated that variation in the D-loop region was reasonable for studies among major lineages of Coregonines (e.g., whitefishes versus ciscoes). However, initial work within the cisco group indicated that D-loop variation was insufficient to differentiate lineages among the more closely related Coregonines (e.g., lake ciscoes versus shortjaw ciscoes). Thus, this study was conducted to develop basic techniques to be used in subsequent mtDNA sequencing studies of cisco taxa, especially those designated to be at some level of risk. The aims of this study were to: 1) design appropriate primers for PCR amplification of Coregonine mtDNA genomes, and 2) test their suitability of amplifying various Coregonines by amplifying the mitochondrial genomes of Coregonines chosen to represent all major lineages within the group.

MATERIALS AND METHODS

Polymerase chain reaction primers were designed using MacVector 7.0 (Accelrys Inc., Madison, WI.), based upon the *Coregonus lavaretus* mitochondrial genome (Genbank accession no. NC_002646) as a template (Miya and Nishida, 2000). When designing the primers with the MacVector program, the following constraints were applied based upon established rules (Baumforth *et al.*, 1998, Kampke *et al.*, 2001, and Rychlik, 1995). Primers were to be free of any significant complementarity and secondary structures such as hairpin loops. Desired primer length was between 18-24 nucleotides, with a similar GC ratio to that of the target (~50% GC). Primer melting temperatures for the two primers were to be as similar to one another as possible, and unique within the target DNA.

The mitochondrial genomes for *Coregonus lavaretus*, *Oncorhynchus mykiss* (Zardoya, 1995), *Salvelinus fontinalis* Mitchill (Dorion *et al.*, 2002), *Salvelinus alpinus* (Dorion *et al.*, 2002),and *Salmo salar* (Hurst *et al.*, 1999) were aligned using MacVector. Highly conserved regions among the aforementioned teleosts were identified as being preferable for the binding locations of PCR primers. Once the primers were chosen, they were again analysed using MacVector to determine whether they could potentially bind to other locations along the mitochondrial genomes of the aforementioned Salmonids. Any primers exhibiting the ability to bind to and amplify sequences other than the desired targets were rejected. These primers were then tested on representative fish species (Appendix 1).

The majority of the primer pairs had an optimal annealing temperature between 50-60 °C. However, the annealing temperatures actually used to amplify the PCR products were not always those determined by the MacVector program to be optimal. The primer annealing temperatures were altered to accommodate for

maximum use of the 40 block thermal cyclers. This allowed for multiple reactions using various primer pairs to be carried out in a single thermal cycler, greatly reducing the need for multiple thermal cyclers and time. In each case, the annealing temperature was rounded off within a five degree range from the optimal temperature.

The primers were manufactured by Invitrogen Canada Co. (Burlington, ON.) as oligonucleotides of standard desalted deoxynucleotides which are lyophilized, then resuspended in sterile distilled water. Total DNA (mitochondrial and genomic) was isolated from tissue[1] using DNeasy tissue kits (Qiagen, Mississauga, ON.) as per manufacturer's recommendations, and stored at –20°C. Polymerase chain reaction mixtures were prepared containing 0.8 mM forward and reverse primers, 0.1 mM dNTP (except where otherwise indicated in Appendix 2), 1 X *Taq* polymerase buffer with 1.5 mM $MgCl_2$ (Roche Diagnostics, Laval, QC.), 10-60 ng of DNA, and 0.5 U *Taq* polymerase (Roche Diagnostics, Laval, QC.) in a 100 µl reaction volume. Amplification of mtDNA was performed in a Techne Genius thermal cycler (Fisher Scientific, Ottawa, ON.) with heated lid as per the thermal cycling parameters in Appendix 2. Successful amplification of the various mtDNA portions was verified by running 5 µl of the PCR product and 1 KB plus ladder (Invitrogen Canada Co., Burlington, ON.) on a 1.0% agarose gel, stained with ethidium bromide (1 mg/ml) and photographed on an ultraviolet transilluminator with Polaroid film.

RESULTS AND DISCUSSION

Polymerase chain reaction primers were designed to accomplish the following goals: 1) to amplify gene coding regions of the Coregonine mtDNA in their entirety, 2) complement existing mtDNA primers (Bernatchez and Danzmann, 1993, Miller *et al.*, 1998, Nielsen *et al.*, 1998) currently used in the FWI fish genetics programs, and, 3) result in mtDNA PCR products that would be suitable for using as templates in automated sequencing.

Since one of the most important factors affecting the quality of PCR is the choice of primers, a few basic parameters were adhered to when designing the primer pairs. The primer should only bind to the target site and not hybridize at any other site. This would result in non-specific amplification, a potential mixed pool of PCR products, and a greatly reduced amount of desired product (Rychlik, 1995). The primers must also be free of significant complimentarity at their 3' ends otherwise primer-dimers may occur, which could lead to a decrease in product due to a lack of primers. Another feature to be avoided is the potential formation of a hairpin loop by primers. Should this occur the primer would become unavailable to bind to the target site. As a result, an uneven primer ratio would become established within the reaction, and assymetrical PCR would occur, or worse yet, no product at all would result (Rychlik, 1995).

Although degenerate primers are just as easy and cheap to produce as unique primers, the design of such primers ~~were~~ was selected against. When degeneracy is too high, unrelated sequences may be amplified as well as the desired sequence (Linhart and Shamir, 2002). By avoiding PCR primers that are degenerate, a second purpose

[1] Tissues can be muscle, fin clips, adipose fin, or any tissue removed from either live-released or dead-sampled fish and preserved frozen, in ethanol, or in a NaCl-saturated 20% dimethyl sulphoxide (1:2 v/v).

the PCR primers may serve is to function as sequencing primers in automated sequencing reactions, since PCR and sequencing primers share many characteristics (Gerischer and Durre, 2001).

An overview of primer locations and the corresponding areas amplified is given in Figure 2, and a more detailed map of primer locations with respect to gene-coding regions is shown in Figure 3. The underlined bases indicate where the primers are located, and not necessarily their exact sequences. Since only the plus strand is shown, reverse primers are the reverse and complement of those bases underlined. Also, individual mismatches between bases underlined and the primers listed in Appendix 2 occur as the depicted sequence is that of *Coregonus lavaretus* and the primers were designed with the intent of amplifying a variety of Coregonines.

Due to the number of existing primers for the D-loop and surrounding regions (Bernatchez and Danzmann, 1993, Miller *et al.*, 1998), no attempt was made to design primers to amplify an intact D-loop region. Rather, primers were designed that by default were able to amplify two separate yet overlapping regions of D-loop. This was done for two reasons. First, the D-loop is often amplified in its entirety using the CytB and HN20 primers with only limited success among different samples and different species. Second, these two primers result in a PCR product of an additional 500 bases that is already amplified when targeting the 12S rRNA region, one of the two separate yet overlapping regions of D-loop. If the D-loop and the 12S rRNA gene are each to be amplified for sequencing purposes, the redundant 500 bases amplified will result in an unnecessary overlap, resulting in both wasted supplies and time.

As previously mentioned, the actual annealing temperatures for the PCR primer pairs employed in the thermal cycling parameters was not always that determined to be optimal by the MacVector program. In the interest of maximizing space in the thermal cyclers, as well as time, the annealing temperatures were "rounded off". No difference was observed between the optimal annealing temperatures and those actually used (data not shown). This allowed for multiple polymerase chain reactions, with different PCR primer pairs to be performed in the same thermal cycler, significantly reducing the need for multiple machines.

The mitochondrial genome was successfully amplified with all of the primers for all Coregonines examined with the exception of *Coregonus muksun* (Table 1). The ND5, ND6, ATPase 6, ATPase 8, and COII regions were not successfully amplified for *C. muksun*. This may be due to the quality of the DNA samples for this fish rather than the primers not being able to bind and amplify through these regions, as the ND5/ND6 primers were able to produce a PCR product for the more distantly related *Salvelinus alpinus*. Wherever possible, primers resulted in PCR products that overlapped by at least fifty base pairs, which will facilitate the alignment of sequences obtained for the different mtDNA fragments.

CONCLUSION

Sequencing of mtDNA for studies investigating taxonomic distinctness, identification and interrelationships requires adequate amounts of DNA to be targeted and amplified using the polymerase chain reaction. For Coregonine fishes, the primers designed and tested herein allow for the successful amplification of the entire mitochondrial genome, and thus provides the basis for such studies.

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Table 1. Success of PCR amplification for taxa examined. For an explanation of the taxon acronyms used, refer to Appendix 1.

Taxon	12S rRNA partial D-loop	16S rRNA	ND1	ND2	COI	COII	COIII	COII & COIII ATPase 6&8	ND 3&4	ND 5&6	cyt *b* partial D-loop
ARCS Baicalensis	+	+	+	+	+	+	+	+	+	+	+
PELD	+	+	+	+	+	+	+	+	+	+	+
LVRT Pidschian	+	+	+	+	+	+	+	+	+	+	+
RSKA	+	+	+	+	+	+	+	+	+	+	+
LKWF (Mississippian form)	+	+	+	+	+	+	+	+	+	+	+
LKCS	+	+	+	+	+	+	+	+	+	+	+
LKWF (Beringian form)	+	+	+	+	+	+	+	+	+	+	+
SJCS	+	+	+	+	+	+	+	+	+	+	+
LSCS	+	+	+	+	+	+	+	+	+	+	+
BDWF	+	+	+	+	+	+	+	+	+	+	+
BRCS	+	+	+	+	+	+	+	+	+	+	+
ARCS	+	+	+	+	+	+	+	+	+	+	+
MUKS	+	+	+	+	+	-	+	-	+	-	+
ARCS (Pollan)	+	+	+	+	+	+	+	+	+	+	+
ARCH	+	+	+	+	+	+	poor	-	+	+	+
BVCS	+	+	+	+	+	+	+	+	+	+	+

"Poor" designation indicates weak band intensity (amplification) as revealed by ethidium bromide staining of PCR products run on a 1% agarose gel. The "+" symbol indicates successful amplification of the coding regions (outer strand) of mtDNA as shown in Figure 1. The "-" symbol indicates unsuccessful amplification of the coding regions (outer strand) of mtDNA.

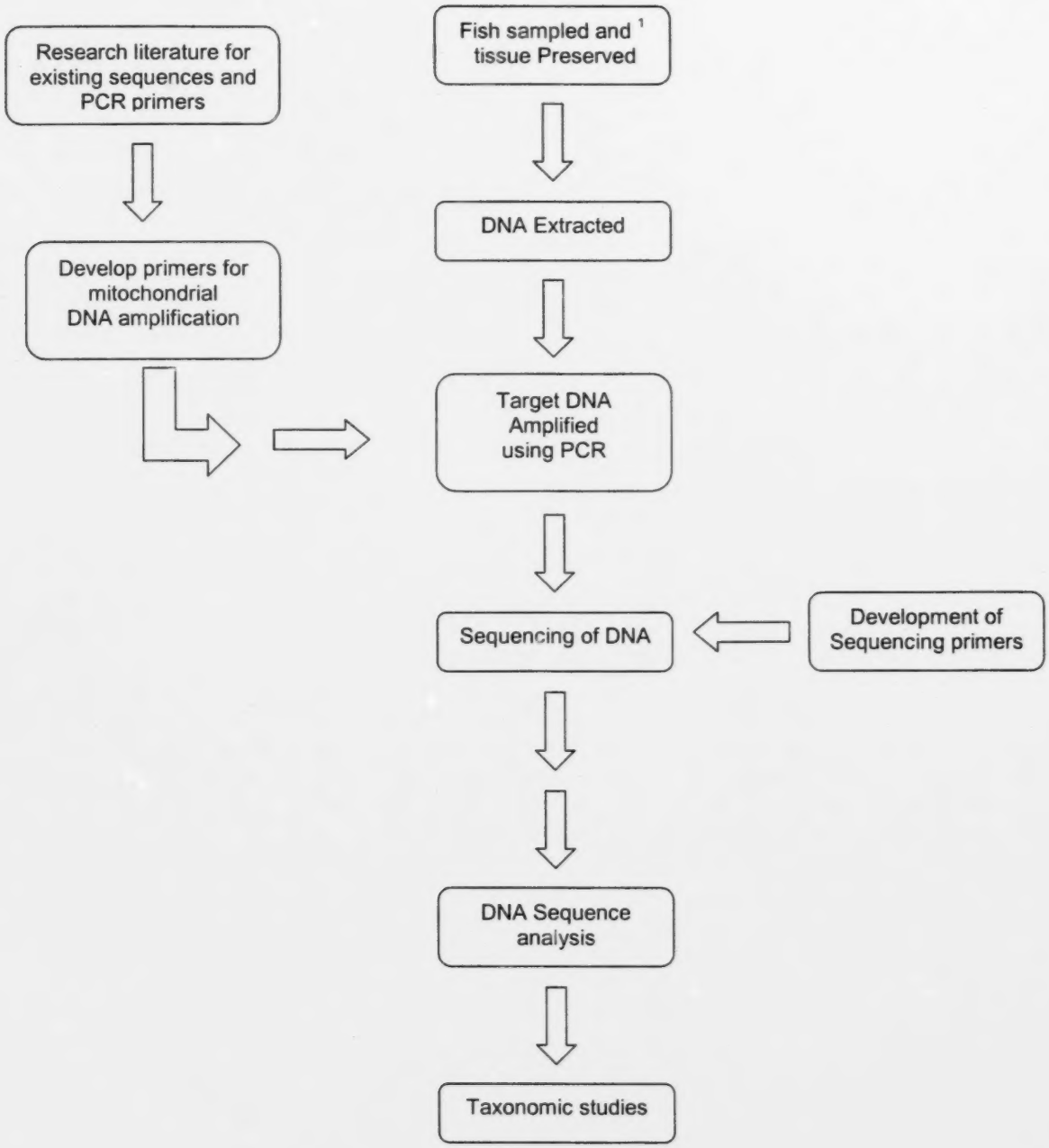
Research literature for existing sequences and PCR primers
Develop primers for mitochondrial DNA amplification
Fish sampled and [1] tissue Preserved
DNA Extracted
Target DNA Amplified using PCR
Sequencing of DNA
Development of Sequencing primers
DNA Sequence analysis
Taxonomic studies

Figure 1. Flow diagram depicting the steps leading to direct sequencing of mitochondrial DNA to obtain taxonomic information. Activity done in this report includes the extraction of DNA, development of PCR primers, and the amplification of mtDNA using PCR.

[1] Tissues can be muscle, fin clips, adipose fin, or any other tissue removed from either live-released or dead-sampled fish and preserved frozen, in ethanol, or in a NaCl-saturated 20% dimethylsulphoxide solution at 1:2 v/v.

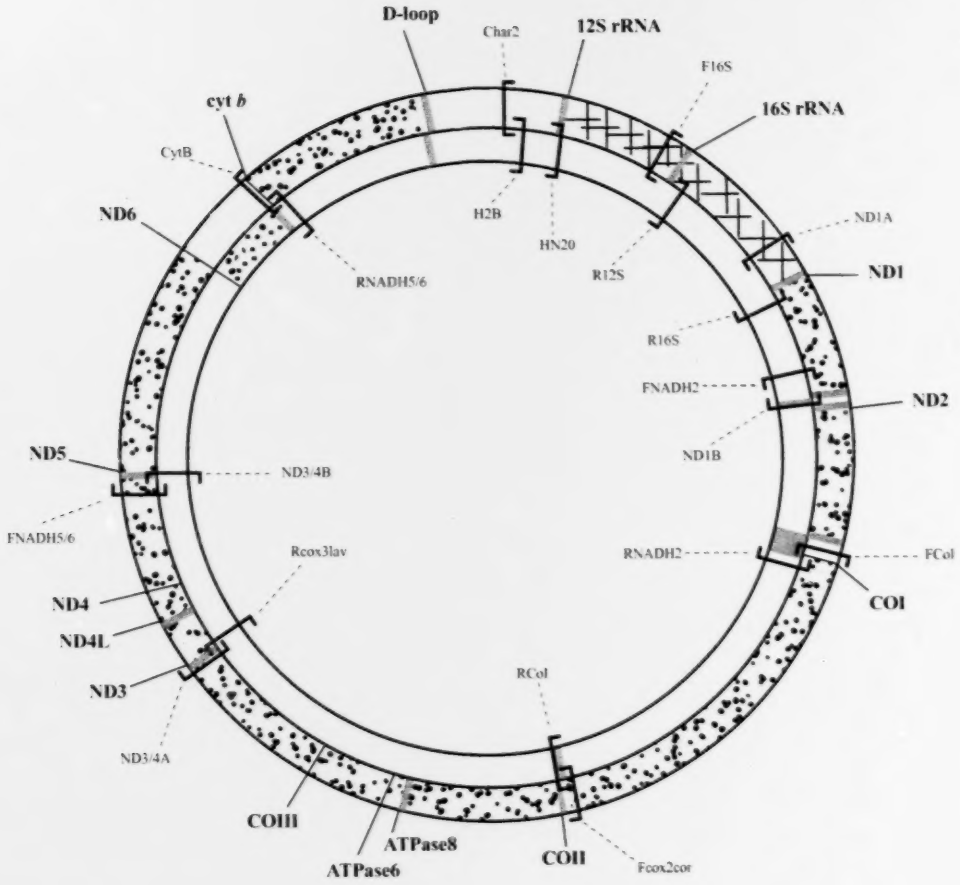

Figure 2. An overview of the mitochondrial DNA of Coregonines (figure not to scale). Gene-coding regions are speckled, rRNA coding regions are cross hatched. tRNA coding regions are gray, and non-coding regions are white. Primers are depicted along the strand and location to which they bind. Primer names are in the non-bold, smaller font (dashed lines); and gene names are in the bold, larger font (solid lines). Forward primers are indicated by "[", and reverse primers by "]". 12S and 16S rRNA are the 12S and 16S ribosomal RNAs; ND1-6 and 4L are the NADH dehydrogenase subunits 1-6 and 4L; COI-III are the cytochrome oxidase subunits 1-3; ATPase6 and 8 are the ATPase 6 and 8 subunits; cyt *b* is cytochrome *b*; and D-loop is the control region.

Figure 3. Locations of primers used in this study, and corresponding gene-coding regions with respect to the *Coregonus lavaretus* mitochondrial genome +strand (Miya and Nishida, 2000). Start of gene-coding regions is indicated by "[" and "]" indicates the end. Gene names are given at the start of the gene, except for ND6 which is located on the complement strand. Primer locations are underlined, and primer names are written above in bold font with arrows indicating direction of primer amplification. Underlined bases are not identical to the primer sequences used in this study; for proper primer sequences, refer to Appendix 2. Primers amplifying in the reverse direction are in most cases, complementary to the –strand. Numbers at the right indicate the position number of the immediately following base in the *Coregonus lavaretus* mtDNA genome.

```
                    ← HN20
gctggcgtag cttaactaaa gcataacact gaagctgtta agatggaccc tagaaagtcc      61
          12S rRNA
cgcaggca[ca aaggcttggt cctgacttta ttatcagctt taactgaact tacacatgca     121
agtctccgca ctcctgtgag gatgccctta atcccctgcc cggggacgag gagctggcat     181
caggcacgcc ccggcagccc aagacgcctt gctaagccac acccccaagg aaactcagca     241
gtgatagata ttaagctata agcgaaagct tgacttagtt aaggttaaga gggccggtaa     301
aactcgtgcc agccaccgcg gttatacgag aggccctagt tgataatcac cggcgtaaag     361
agtggttagg aattatattt aataaagccg aacacccccc tggctgtcat acgcacctgg     421
gggcacgaag ccccactgcg aaagcagctt taatcaccac ctgaacccac gacagctatg     481
atacaaactg ggattagata ccccactatg cctagccgta aactttgatg gaaacataca     541
actaacatcc gccagggaac tacaagcgcc agcttaaaac ccaaaggact tggcggtgcc     601
tcagacccac ctagaggagc ctgttctaga accgataacc cccgttcaac ctcaccacct     661
cttgttttcc ccgcctatat accaccgtcg tcagcttacc ctgtgaagga tttatagtaa     721
gcaaaatggg catgacccaa aacgtcaggt cgaggtgtag cgcatggggt gggaagaaat     781
gggctacatt ctctaaatta gagcattacg aaccacgctg tgaaaccagc gtccgaaggt     841
ggatttagca gtaaacagaa agcagagagt tctcttgaaa ctggctctga ggcgcgcaca     901
caccgcccgt cactctcccc aagttcaatc tacccttcta actaagaagt taaccgaaca     961
aaggggaggc aagtcgtaac atggtaagtg taccggaagg tgcacttgga ataac]cagag    1021
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← Rcox2lav ATPase 6

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gtaagcaaac actacaagta tccccccaa ataaatcaag aatagtacta aagacaagaa        14161
cgaccccca tgccccacca aaacaccaca gcccacgccc gctgccacta ccaaccccag       14221
ggcagcaaag tagggagcag gatttgatgc aacagctaca agacccaaaa ccaaccccaa      14281
                          ND6 (complement)
tagaaataaa gacacaagat aagtcat]aat tcctgctcgg actctaaccg aaactaatga     14341
          CYTB →                                cytb
cttgaaaaac caccgttgtt attcaactac aagaaccta[a tggctaacct ccgaaaaacc     14401
          ← RNADH5/6
cacccctcc taaagattgc taatgacgca ctagtcgatc ttccagcgcc ctcaaacatc       14461
tcagtgtgat gaaactttgg ctcacttctg ggcttatgtt tagccaccca aattctcaca       14521
ggacttttcc tggccatgca ctacacttct gacatctcaa cagccttctc ctccgtatgc       14581
catatttgcc gagatgtcag ctacggctga cttatccgaa atattcacgc caatggagca       14641
tctttcttct ttatctgcat ttatatacac attgcccgag gactttacta tggctcctac       14701
ctatacaaag aaacctgaaa tattggggtt gtccttctac tccttacaat aataactgcc       14761
tttgtgggct acgttcttcc atgaggacaa atatctttct gaggtgcaac agtcatcaca       14821
aacctccttt ctgccgtgcc ctacgtaggg ggcgcccttg tgcagtgaat ctgaggtggg       14881
ttttccgtag ataatgccac cctaacacgg ttttttgcct ttcacttctt attcccattt       14941
```

gttattgcag ctgcaacagt catccacctc ctcttccttc atgaaactgg gtctaacaac 15001
ccagcaggga tcaactctga tgccgataaa atctcattcc acccctactt ctcatacaaa 15061
gacctgttgg gatttgtggc tatactacta ggactaacat ccttggcact atttgcaccc 15121
aacctcttgg gggacccaga taattttaca ccggccaacc cactagtcac cccgccccac 15181
atcaagcctg agtgatactt cctcttcgcc tacgcaatcc tacgatcgat ccccaacaag 15241
ctaggcgggg tcctcgccct gctattctct atcctcgtac tcatggtcgt ccccattcta 15301
catacctcta agcagcgagg actaaccttc cggcccctta cgcaattctt attctgaacc 15361
ctagtagcag acatgctcat cctcacctga attggcggta tacctgtaga acacccccttc 15421
atcatcatcg gccaagtcgc ctcagttatc tacttcacca tcttcctagt cttggccccc 15481
ttagccggat gagctgagaa taaagccctc gaatgagcct] gccctagtag ctcagtgtaa 15541
gagcgccggt cttgtaatcc ggaggccgga ggttaaaccc ctccctagtg ctcagagaga 15601
ggagatttta actcccaccc ttaactccca aagctaagat tctaaattaa actaccctct 15661

D-loop

g[acgccgcag tgtacatggt aaatcatata tccccataca gtgtgtgtat attacaccac 15721
tatgtataat attgcatatt atgtactgac ccatatatta ttattgcacg taggtagtac 15781
atcctatgta ttatcaacat aagtgatttt aagccctcat acatcagtac tgttccaagg 15841
tttacataag caagactcgg ataatcacca acggaaccgt tctaacctga ttaattgcta 15901

Char2 →

aacaacaaac ctccaactaa cacgggctcc gtctttaccc accaaatttc agcatcggtc 15961
ccgtttaatg tagtaagaac cgaccaacga tttattagta ggcatactct taatgatggt
16021 ← **H2B**
cagggtcaaa tatcgtatta ggtagcatct cgtgaattat tacttgcatc tggttcctat 16081
ttcatgggct atccttaaga aaccacccccc tgaaagccga atgtaatgca tctggttaat 16141
ggtgtcaacc ttactgttcg ttacccacct agccgggcgt tctcttatat gcatagggtt 16201
ctccttttttt ttttttcctt tcagcttgca tatacaagtg cacaccgaga agtctaacaa 16261
ggtcgaacta gatcttggtc tccagcggac ccaataataa tggcggaatg atattctata 16321
aagaattgca taattgatat caagtgcata aggtcagttt ctttcctcac agatacctaa 16381
gatctccccg gcttttgcgc ggctaaaccc ccctaccccc ctacgctgag cgatccttat 16441
tattcctgtc aaaccccaaa accaggaagt ctcgatagcg ctattaccca tcaaaccata 16501
cattaacaaa ctttggcacc gacaatccta ttatcaaagc caccccttaa ttaaagtgta 16561
cactaaaact ttttattata cattaataaa ctttattact tacaaacttt ggcaccgaca 16621
atcctattat caaagccacc ccttaattaa agtatacatt aataaaattt ttgttatact 16681
taacaaactt tggcaccgac aaccctatca tgaaggccac tcctggttaa aatatat]

Appendix 1. Fish taxa used in this study.

Acronym	Nominate taxon (common name)	Fish ID[1]	Sample Location	Location codes[1]
LKCS	*Coregonus artedi* (Lake cisco)	45769 45770	Lake Athapapaskow (S. Basin), Manitoba	00-21-02
SJCS	*Coregonus zenithicus* (Shortjaw cisco)	46317 46318	George Lake, Manitoba	00-50-01
LSCS	*Coregonus sardinella* (Least cisco)	43297 43298	North Killeak Lake, Alaska	96-24-05
ARCS	*Coregonus autumnalis baicalensis* (Baikal cisco)	44418 44419	Lake Baikal, Russia	98-19-01
ARCS	*Coregonus autumnalis* (pollan) (Irish pollan)	36303 36319	Loch Neagh, Ireland	91-29-01
ARCS	*Coregonus autumnalis* (Arctic cisco)	32051 32052	Atkinson Point, Northwest Territories	88-38-01
LVRT	*Coregonus lavaretus pidschian* (European whitefish)	71080 71081	Kuluymbe River, Northern Siberia	00-61-01
PELD	*Coregonus peled* (Peled)	71270 71271	Khantaiskoe Resevoir, Northern Siberia	00-60-01
LKWF	*Coregonus clupeaformis* Mississippian form (Lake whitefish)	28308 28309	Big Black River, Manitoba	89-30-01
LKWF	*Coregonus clupeaformis* Beringian form (Lake whitefish)	37215 37216	Klutina River, Alaska	91-12-02
BDWF	*Coregonus nasus* (Broad whitefish)	41306 41307	Little Chicago, Mackenzie River, Northwest Territories	95-05-01
MUKS	*Coregonus muksun* (Muskun)	M1 M2	Middle Ob River, Siberia	
BRCS	*Coregonus laurettae* (Bering cisco)	36812 36813	Yukon River, Alaska	91-32-01
RSKA	*Coregonus lavaretus* Reeska form (Reeska)	36071 36702	Lake Inari, Finland	91-26-01
BVCS	*Prosopium gemmifer* (Bonneville cisco)	47289 47290	Bear Lake, Utah	00-69-01
ARCH	*Salvelinus alpinus* (Arctic char)	70444 70445	Taserssuit Lake, Greenland	00-40-01

1. Fish identification (ID) number and location code cross-reference the sample to the (FWI AFEAR) internal fish sample database.

Appendix 2. Thermal cycling programs for amplification of mitochondrial DNA[a].

1. 12S rRNA, partial d-loop
   Forward primer: CHAR2 5'-CAA AAC TCC AAC TAA CAC GG-3'
   Reverse primer: R12S 5'-AAC CTC TGA GAA AAA GGG C-3'
   Thermal cycling program: -2 min. @ 95°C
   -30 sec @ 94 °C, 35 sec @ 50 °C, 2 min 30sec @ 70 °C for 30 cycles
   Results in a product ~2000 bases in length.

2. 16S rRNA
   Forward primer: F16S 5'-ACA CCG AGA AGA CAT CCG TG-3'
   Reverse primer: R16S 5'-AAC CTC TGA GAA AAA GGG C-3'
   Thermal cycling program: -2 min @ 95 °C
   -45 sec @ 95 °C, 1 min @ 60 °C, 2 min @ 72 °C for 30 cycles
   Results in a product ~1800 bases in length.

3. ND1
   Forward primer: ND1A 5'-GCC TCG CCT GTT TAC CAA AAA CAT-3'
   Reverse primer: ND1B 5'-GGT ATG GGC CCG AAA GCT TA-3'
   (Nielsen *et al.*, 1998)
   Thermal cycling program: -2 min @ 95 °C
   -45 sec @ 95 °C, 30 sec @ 50 °C, 2min 30 sec @ 70 °C for 8 cycles
   -45 sec @ 95 °C, 30 sec @ 53 °C, 2min 30 sec @ 70 °C for 21 cycles
   Results in a product ~2000 bases in length.

4. ND2
   Forward primer: FNADH2 5'-AAA AGC CGC CCT ACT TAC CG-3'
   Reverse primer: RNADH2 5'-ATC ATA ACG AAG GCG TGG GC-3'
   Thermal cycling program: -2 min @ 95 °C
   -30 sec @ 94 °C, 35 sec @ 55 °C, 2 min 30 sec @ 72 °C for 30 cycles
   Results in a product ~2000 bases in length.

5. COI
   Forward primer: FCol 5'-ATG GGG TTA CAA TCC ACC GC-3'
   Reverse primer: RCol 5'-GTG AGG GAT GTG CCA TTGTAG C-3'
   Thermal cycling program: -2 min @ 95 °C
   -30 sec @ 94 °C, 35 sec @ 55 °C, 2 min 30 sec @ 72 °C for 30 cycles
   Results in a product ~1800 bases in length.

6. COII
   Forward primer: Fcox2cor 5'-CAA GCC AAC CGC ATA ACC-3'
   Reverse primer: Rcox2lav 5'-ACG AGA ATA CCA AGA TGG-3'
   Thermal cycling program: -2 min @ 95 °C
   -45 sec @ 95 °C, 1 min @ 55 °C, 2 min @ 72 °C for 30 cycles
   Results in a product ~1100 bases in length.

7. COIII
   Forward primer: Fcox3cor 5'-CCT TTA CCT ACA AGA AAA CG-3'
   Reverse primer: Rcox3lav 5'-CAG AAA GAA ACA GTA GCC AG-3'
   Thermal cycling program: -2 min @ 95 °C
   -45 sec @ 95 °C, 1 min @ 55 °C, 2 min @ 72 °C for 30 cycles
   Results in a product ~950 bases in length.
   Note: Can alternatively use the following program (8) to amplify Cox2, Cox3 and everything in between.

8. COII, COIII, ATPase6, ATPase8
   Forward primer: Fcox2cor 5'-CAA GCC AAC CGC ATA AAC-3'
   Reverse primer: Rcox3lav 5'-CAG AAA GAA ACA GTA GCC AG-3'
   Thermal cycling program: -2 min @ 95 °C
   -45 sec @ 95 °C, 1 min @ 55 °C, 2 min @ 72 °C for 30 cycles
   Note: Must use 4 *u*l of dNTPs/sample to be amplified due to product length.
   Results in a product ~2700 bases in length.

9. ND3, ND4
   Forward primer: ND3/4A 5'-TTA ATA CGT ATA AGT GAC TTC CAA-3'
   Reverse primer: ND3/4B 5'-TTT TGG TTC CTA AGA CCA ATG GAT-3'
   (Nielsen *et al.*, 1998)
   Thermal cycling program: -2min @ 95 °C
   -45 sec @ 95 °C, 30 sec @ 50 °C, 2min 30 sec @ 70 °C for 8 cycles
   -45 sec @ 95 °C, 30 sec @ 53 °C, 2 min 30 sec @ 70 °C for 21 cycles
   Results in a product ~2300 bases in length.

10. ND5, ND6
    Forward primer: FNADH5/6 5'-CTT ATC CAC CGA GAG AAG TCT GTT G-3'
    Reverse primer: RNADH5/6 5'-TGC GTC ATT AGC AAT CTT TAG GAG-3'
    Thermal cycling program: -2min @ 95 °C
    -30 sec @ 94 °C, 35 sec @ 55 °C, 2 min 30 sec @ 70 °C for 30 cycles
    Note: Must use 4 *u*l of dNTPs/sample to be amplified due to product length.
    Results in a product ~2600 bases in length.

11. cyt *b*, partial D-loop
    Forward primer: CytB 5'-GAA AAA CCA YCG TTG TWA TTC AAC T-3'
    (Miller *et al.*, 1998)
    Reverse primer: H2B 5'-GCC AGG AAT AAT TCA CGA G-3'
    Thermal cycling program: -2min @ 95 °C
    -1 min @ 94 °C, 1 min @ 48 °C, 1 min 30 sec @ 72 °C for 32 cycles
    Results in a product ~1700 bases in length.

Note: The d-loop has been amplified in two separate yet overlapping segments (1 & 11), but can also be amplified intact using the following program (12) with limited success.

12. D-loop
    Forward primer: LN20 5'-ACC ACT AGC ACC CAA AGC TA-3'
    Reverse primer: HN20 5'-GTG TTA TGC TTT AGT TAA GC-3'
    (Bernatchez and Danzmann, 1993)
    Thermal cycling program: -4min @ 95 °C
    -1 min @ 94 °C, 1 min @ 48 °C, 1 min 30 sec @ 72 °C for 32 cycles
    Note: Must use 4 *u*l of dNTPs/sample to be amplified due to product length.
    Results in a product ~1150 bases in length.

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[a] All nucleotides are given according to their IUB codes (IUB nomenclature committee, 1985).